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ORIGINAL ARTICLE – ENDOCRINE TUMORS

In Vitro Chemoresistance Testing in Well-Differentiated Carcinoid Tumors

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ABSTRACT

Background. Well-differentiated, "typical" carcinoid tumors traditionally have a very poor response to chemotherapy. We hypothesized that tumor specimens from well-differentiated carcinoid tumors would be highly resistant to the effects of chemotherapy when tested against a variety of antineoplastic agents in vitro.

Methods. Ninety-eight typical carcinoid specimens were surgically harvested, cultured, and tested against antineoplastics in vitro. ^{3}H-Thymidine incorporation was used to assess the percentage of cell-growth inhibition (PCI) of tested specimens. PCI was used to determine if specimens had extreme drug resistance (EDR), intermediate drug resistance (IDR), or low drug resistance (LDR) to each reagent against which they were tested.

Results. Seventy specimens generated results. Each was tested with an average of six drugs. The mean proportions of drugs classified as LDR, IDR, and EDR were 0.48 (range 0–1), 0.34 (range 0–1), and 0.18 (range 0–0.80), respectively. The mean numbers of drugs per specimen exhibiting LDR, IDR, and EDR chemoresistance were 2.7, 2.1, and 1.2, respectively. 57 of 70 specimens (81%) had LDR to at least two drugs. 5-FU had the highest frequency of low chemoresistance at 69%, followed by doxorubicin at 67%. Low in vitro resistance to chemotherapeutics was prevalent among typical carcinoids, while EDR was comparatively infrequent.

Conclusions. This implies that there may be less clinical chemoresistance and more chemosensitivity among typical carcinoid tumors than clinical trials have previously revealed. These findings warrant additional investigations assessing the response of carcinoid tumors to assay-guided chemotherapy regimens.

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Chemotherapeutic options for patients with slowly proliferating, well-differentiated (typical) carcinoid tumors are limited.[1] Clinical trials have demonstrated response rates to single-agent chemotherapy that are approximately 20%, and multiagent chemotherapy response rates are almost always less than 40%.[2] Moreover, these responses are often short lived and rarely translate to prolonged survival.[3,4] In contrast, responses of rapidly proliferating, poorly differentiated (atypical or small cell-like) carcinoid tumors to chemotherapy are quite high, but the duration of response is extremely short.[5] One potential explanation for the lack of efficacy of antineoplastic agents in the treatment of typical well-differentiated carcinoids is that their cells are too slow growing to be affected by cytotoxic agents that influence only actively dividing tumor cells. In general, carcinoid tumors labeled as slowly proliferating, well-differentiated, or "typical" have proliferative indices (Ki-67) of less than 2%. Little is known about in vitro chemoresistance testing of carcinoids or other well-differentiated neuroendocrine tumors (NETS). However, based on the

clinical unresponsiveness of well-differentiated carcinoid tumor to chemotherapy, we hypothesized that tumor specimens from these well-differentiated carcinoid tumors would be highly resistant to the effects of antineoplastic agents when tested against a variety of antineoplastic agents in vitro.

MATERIALS AND METHODS

From July 2005 until June 2007, the Louisiana State University (LSU) Neuroendocrine Tumor Group (LSUHSC, New Orleans, LA) harvested 72 carcinoid specimens from 52 patients undergoing surgical cytoreduction. At surgery, specimens were subdivided. One portion was sent for immunohistochemical analysis and the other section for drug resistance testing. Sections destined to undergo drug resistance testing were placed aseptically into a vial of chilled RPMI media and transported by overnight courier to a commercial laboratory.

From July 2005 to March 2006, specimens were sent to Oncotech, Inc. in Irvine, CA, for drug resistance testing. During this time, immunohistochemical analysis was performed by a LSU pathologist. From April 2006 to June 2007, specimens were sent to Genzyme, Inc. in Los Angeles, CA, for both drug resistance testing and immunohistochemical analysis. No identifying patient information was collected or stored. Additionally, during this time, the neuroendocrine tumor group at Mount Sinai Hospital (Mount Sinai Medical School, New York, NY) collected 26 typical abdominal NETS for similar analysis. These specimens were sent to Oncotech, Inc. in Irvine, CA, for drug resistance testing, and two Mount Sinai Pathologists (J.S., M.I.) performed the immunohistochemical analysis on these specimens. Data from these tumors were gathered by Mount Sinai researchers and then shared with LSU researchers. LSU researchers then combined the Mount Sinai data with their own data for analysis. Internal Review Board approval was present, and no identifying patient information was collected or stored. Thus, this report describes the evaluation of 98 freshly collected NETS for proliferative index assessment and chemoresistance testing.

Chemoresistance Assay

Chemoresistance assays performed on specimens harvested at Mt. Sinai were done by Oncotech, Inc. The chemoresistance assays on specimens harvested at LSU were performed by Oncotech, Inc. from July 2005 to March 2006, and by Genzyme, Inc. from April 2006 to June 2007. Both companies use a similar methodology to perform the Drug Resistance Assay based on technology developed by Kern and Weisenthal.[6]

Briefly, viable cells were suspended in soft-agarose media, and single-agent antineoplastic reagents were added at doses greater than their clinically achievable plasma concentrations.[6] After incubation for 72 h, 5 μCi of tritiated thymidine was added to each well, and cultures were incubated for an additional 48 hours. Cells were subsequently lysed, and the contents were harvested onto glass fiber filters. Cellular proliferation was determined by tritiated thymidine–DNA incorporation and expressed as counts per minute. Results were reported as percent cell-growth inhibition of the individual drug compared with media-exposed control cultures correcting for positive control counts per minute. For each agent tested, the median percent cell-growth inhibition (PCI) and standard deviation (SD) result from a patient's tumor cell culture are compared with the median PCI and SD of the entire historic population database tested against that drug. Tumors were graded as having low, intermediate, or high drug resistance. Tumors exhibiting PCI values one SD above the median are considered to express low drug resistance (LDR); those with PCI values between the median and one SD below the median are categorized as having intermediate drug resistance; and tumors with PCI values one SD below the median are categorized as having high drug resistance.[7]

Chemoresistance Score

A scale ranging from 1 to 3 was used to develop a mean chemoresistance score for each antineoplastic agent tested. Low chemoresistance was assigned a value of 1, intermediate chemoresistance was assigned a value of 2, and high chemoresistance was assigned a value of 3. The mean chemoresistance score of each drug was determined by taking the average of the individual specimen chemoresistance scores.

For example, topotican was tested against two specimens. One specimen was found to have intermediate chemoresistance against topotican (score = 2), and another specimen was found to have low chemoresistance against this reagent (score = 1). Thus, topotican's mean chemoresistance score was 1.5. A mean chemoresistance score closer to one indicated a greater incidence of low chemoresistance, while a score closer to three indicated a greater incidence of high chemoresistance.

Statistical Analyses

Both the patients' Ki-67 value and the proportion of drugs classified as having low chemoresistance were of interest in this study. To test for the presence of significant differences in these two outcome variables, a three-factor analysis of variance (ANOVA) model was used. The three

factors of interest were gender (male or female), primary tumor location (foregut, midgut, hindgut, or unknown), and specimen source (primary, liver, nodal, or other). Interaction effects were not tested due to the relatively large number of factor levels and the relatively small number of patients. The Tukey–Kramer test was used for post hoc comparisons among the levels of the three factors. Additionally, the comparison of chemoresistance scores for three specific drugs (5-FU, doxorubicin, and cisplatin) was of interest. A one-way ANOVA with drug as the factor variable was used to test for significant differences among the three drugs. Results were considered significant at the 0.05 nominal level. Statistical analyses were performed using SAS version 9.1 and GraphPad Prism version 4.

RESULTS

A total of 98 specimens from 78 patients were harvested and deemed to be "typical" carcinoids based on a Ki-67 proliferative index of no greater than 2%. There were six specimens from four patients whose primary tumors arose in the pancreas, and while these were reported to be carcinoid tumors rather than islet cell tumors of the pancreas, they were excluded from this analysis. Of the 92 remaining specimens, 22 of the tumors did not grow in culture, grew but became infected, had an insufficient quantity of tissue supplied, or had other technical issues that prevented us from being able to determine chemoresistance.

Chemoresistance results were obtained for 70 specimens. Of these, 42 specimens (60%) were from female patients and 28 (40%) were from male patients. Additionally, 15 specimens (21%) each were from liver and nodal sources, 6 (9%) were from the primary tumor, and 34 (49%) were from other sources. There were 49, 6, and 3 specimens (70%, 9%, and 3%) from patients whose primary tumors were located in the midgut, foregut, and hindgut, respectively. There were 12 specimens (17%) from patients with an unknown primary. Means and standard deviations for the Ki-67 values and the proportion of drugs classified as low chemoresistance can be found in Table 1. There were no statistically significant differences present in the mean Ki-67 values among the levels of the three factors, gender, primary tumor location, and specimen source ($F = 0.94$; $P = .4836$). Similarly, there were no statistically significant differences present in the mean proportion of drugs that were classified as low resistance among the levels of the three factors ($F = 0.63$; $P = .7283$).

A total of 23 drugs were tested for chemoresistance in the 70 specimens; however, not every specimen was tested with every drug. On average, each specimen was tested with 6 drugs, although the number of drugs tested ranged from 2 to 11 drugs. The mean proportions of drugs expressing low, intermediate, and high chemoresistance were 0.48 (range 0–1), 0.34 (range 0–1), and 0.18 (range 0–0.80), respectively (Fig. 1). The mean numbers of drugs classified as low, intermediate, and high chemoresistance were 2.7, 2.1, and 1.2, respectively. In addition, there were three specimens in which no drug (0%) was found to have low chemoresistance, and there were 10 specimens in which only one drug was found to have low chemoresistance. There were 21 specimens in which two drugs were found have low chemoresistance, and there were 36 specimens in which three or more drugs were found to have low chemoresistance (Fig. 2).

A total of 23 different drugs were tested at least once. The three most frequently tested drugs were 5-FU,

TABLE 1 Demographics of specimens

	n	Mean (SD) of Ki-67 value	Mean (SD) proportion of low resistance drugs
Gender[a]			
Males	28	0.99 (0.491)	0.50 (0.239)
Females	42	1.06 (0.586)	0.47 (0.251)
Location of primary tumor[a]			
Foregut	6	1.05 (0.589)	0.46 (0.277)
Midgut	49	1.03 (0.560)	0.48 (0.239)
Hindgut	3	1.57 (0.513)	0.24 (0.218)
Unknown	12	0.90 (0.461)	0.57 (0.245)
Source of the specimen[a]			
Primary	6	0.83 (0.755)	0.45 (0.152)
Liver	15	1.20 (0.646)	0.45 (0.264)
Nodal	15	0.87 (0.458)	0.49 (0.256)
Other	34	1.06 (0.491)	0.50 (0.252)

[a] No statistically significant differences were found between the factor levels

SD standard deviation

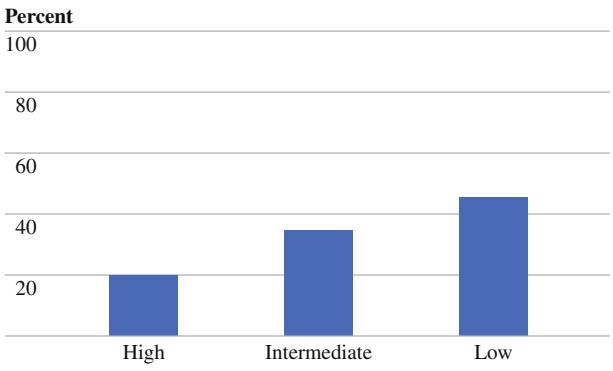

FIG. 1 Chemoresistance profiles of all reagents tested. $n = 423$. A total of 423 chemoresistance profiles were reported for 70 specimens. Each specimen was tested with an average of 6 drugs (range 2–11). The mean percentage of drugs classified as low, intermediate, and high chemoresistance were 48% (range 0–100%), 34% (range 0–100%), and 18% (range 0–80%), respectively

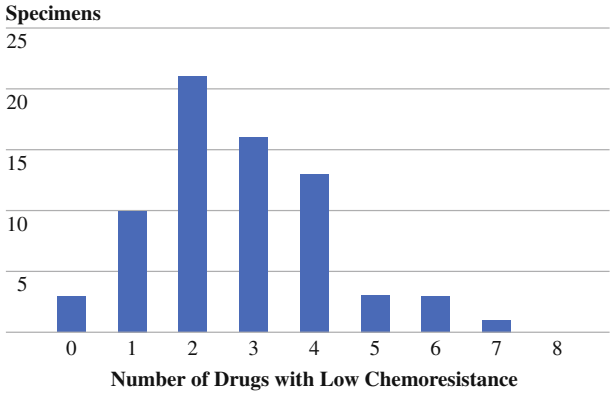

FIG. 2 Number of specimens that had low chemoresistance to multiple drugs. This figure outlines the number of low chemoresistance drugs that were seen per specimen. Although, there were 3 specimens in which no (0%) drug was found to have low chemoresistance, there were 57 of 70 specimens (81%) with at least 2 low resistance reagents

doxorubicin, and cisplatin. The resistance profiles for these drugs, including percentages in each of the three resistance categories as well as means and standard deviations of the resistance scores may be found in Table 2. Of the three drugs, 5-FU had the highest frequency of low chemoresistance at 69%, followed by doxorubicin at 67% and cisplatin at 22%. Similarly, both 5-FU and doxorubicin had low mean chemoresistance scores of 1.4, while cisplatin's score was 2.3 (Fig. 3). Cisplatin's mean chemoresistance score was significantly greater than both 5-FU and doxorubicin's mean scores ($F = 35.54$, $P < .001$). Etoposide exhibited LDR in 31 of 51 specimens (58%), and its mean chemoresistance score was 1.53. Dacarbazine exhibited LDR in 16 of 42 specimens (38%), and its mean chemoresistance score was 1.67. Graphical representation of the mean chemoresistance scores for all 23 of the drugs tested may be found in Fig. 4.

TABLE 2 Drug resistance profiles to frequently tested reagents

Drug	LDR		IDR		EDR		Score	
	n	%	*n*	%	*n*	%	Mean	SD
5-FU	45	69.2	17	26.2	3	4.6	1.4	0.57
Doxorubicin	43	67.2	16	25.0	5	7.8	1.4	0.64
Etoposide	31	58.5	16	30.2	6	11.3	1.5	0.70
Dacarbazine	16	38.1	24	57.1	2	4.8	1.7	0.57
Cisplatin	14	22.2	19	30.2	30	47.6	2.3	0.80
Temozolamide	14	56.0	6	24.0	5	20.0	1.6	0.81
Cyclophosphamide	6	37.5	5	31.3	5	31.3	1.9	0.85
Interferon-α	3	15.0	12	60.0	5	25.0	2.1	0.64

LDR low drug chemoresistance, *IDR* intermediate drug chemoresistance, *EDR* extreme drug chemoresistance, *SD* standard deviation

DISCUSSION

Chemotherapy has not been effective for most patients with well-differentiated carcinoid. 5-FU,[8] doxorubicin,[9] streptozotocin,[8] and dactinomycin[8] have been the most effective single reagents, but these drugs still only yield responses of 13% to 26% as single agents (Table 3). Combination regimens using these reagents have been tested, and they have also failed to yield encouraging results or enhance the duration of responses. A recent ECOG trial of combination chemotherapy randomly assigned patients to receive either 5-FU and streptozotocin (FS) or 5-FU and doxorubicin (FA).[10] Additional cohorts of patients were directly assigned to receive dactinomycin as a single agent. There were 163 patients accrued to the randomized arms who had valid data for analysis. FA and FS therapies were associated with response rates of 13% and 16%, respectively. With dactinomycin, the response rate was approximately 10%. Newer chemotherapeutics have also been shown to be inactive. High-dose paclitaxel yielded one nonsustained partial response among 14 patients with carcinoid.[11] Docetaxel yielded biochemical responses in some, but no radiologic responses in the 21 patients evaluated with this drug.[12] No responses were seen in patients treated with gemcitabine.[13]

We studied the patterns of in vitro chemoresistance of "typical" carcinoid tumors. Because clinically these tumors respond poorly to chemotherapy in vivo, we predicted that their tumor specimens would be extremely resistant to chemotherapy in in vitro drug resistance assays. However, we found that these specimens had extreme drug resistance to only 20% of the drugs against which they were tested, and they had LDR to almost half (45%) of the drugs against which they were tested. The incidence of LDR was more than twice the incidence of extreme drug resistance, and more than 80% of specimens had low chemoresistance to at least two drugs. These results imply that there exists a group of patients with typical carcinoid that are less chemoresistant and more chemosensitive than studies have previously demonstrated.

Two different labs performed the chemoresistance assay for this study. Both used similar techniques as described by Kern et al.,[6] and both labs specialize in this assay. Specimen demographics were similar in both labs (Table 4). The results between the two companies varied when analyzing the chemoresistance profile of one drug, but they were consistent when comparing changes in chemoresistance between two different drugs. For instance, 30% of the specimens tested at Genzyme had low resistance to cisplatin compared with 4% of the specimens from tested at Oncotech. However, results from both companies demonstrated that fewer specimens had low resistance to cisplatin than to 5-FU and doxorubicin. See Fig. 4.

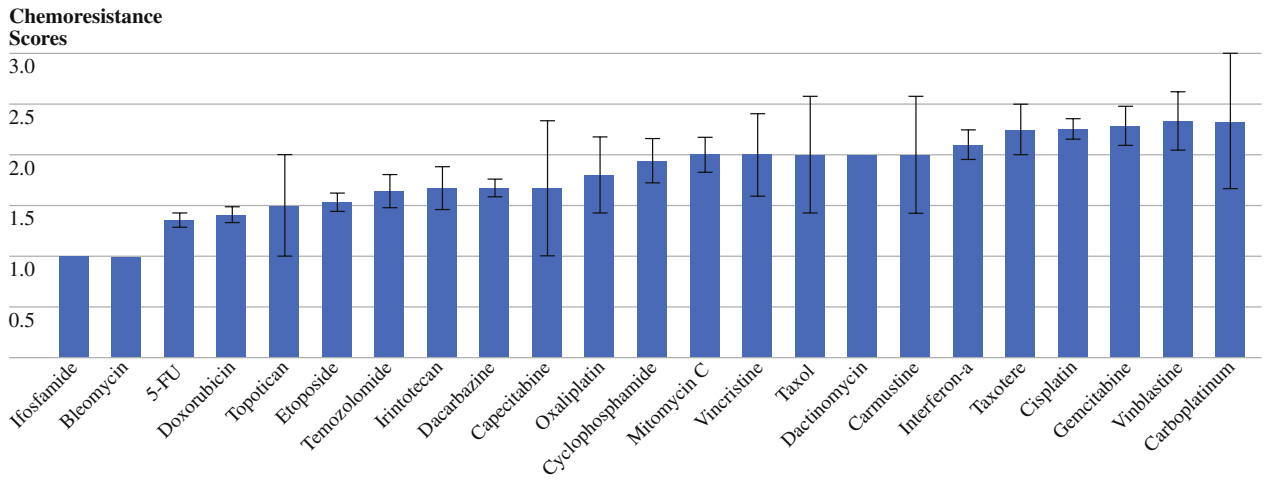

FIG. 3 Mean chemoresistance scores of reagents tested against typical carcinoid tumors. 1, low resistance; 2, intermediate resistance; 3, high resistance. Results are expressed as Mean ± SEM

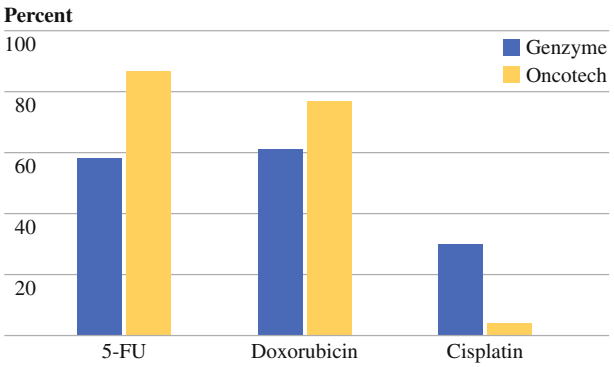

FIG. 4 Percent of specimens with LDR. Comparison of LDR results from two different lab companies. While variations exist in intradrug comparisons, interdrug comparisons were relatively similar

There are inherent problems with in vitro assays including the inability to translate in vitro results directly into in vivo clinical responses. Factors such as individual patient or tumor metabolism, tumor vascular supply, and anatomic permeability barriers affect the interaction between the tumor and chemotherapy clinically, and these factors are not readily reproducible in the laboratory. Despite these challenges, several in vitro assays have been developed in an attempt to predict the response of an individual patient's tumor to a particular chemotherapeutic agent. Such a system could potentially identify the most effective drug with which to treat the patient, and patients could be spared from the side effects of ineffective drugs.

TABLE 3 Selected studies of antineoplastics in patients with carcinoid

Reagent	No. of patients who responded to chemotherapy (%)	Reference
Doxorubicin	7/33 (21), 8/91 (20)	8,9
5-FU	5/19 (26)	8
Dactinomycin	2/15 (13), 2/21 (10)	8,10
Streptozocin	1/6 (17)	8
Gemcitabine	0/9 (0)	13
Paclitaxel	1/14 (7)	11
Docetaxel	0/21 (0)	12
Cisplatin	1/10 (10)	8
Etoposide + cisplatin	0/13 (0)	5
Streptozocin + doxorubicin	4/10 (40), 2/9 (22)	20,21
5-FU + streptozocin	24/104 (23), 12/78 (15)	9,10
5-FU + doxorubicin	11/85 (13)	10
5-FU + doxorubicin + cytoxan + streptozocin	17/56 (30)	22
5-FU + cytoxan + streptozocin	2/9 (22)	22

TABLE 4 Demographics of specimens by drug company

	Genzyme		Oncotech	
	n	%	*n*	%
Gender				
Males	15	35	13	48
Females	28	65	14	52
Location of primary tumor				
Foregut	5	12	1	4
Midgut	31	72	18	66
Hindgut	2	4	1	4
Unknown	5	12	7	26
Incidence of chemoresistance grades				
LDR	117	47	75	43
IDR	102	40	45	26
EDR	32	13	52	30

LDR low drug chemoresistance, *IDR* intermediate drug chemoresistance, *EDR* extreme drug chemoresistance

Von Hoff et al. prospectively compared the response of patients with advanced metastatic cancer to single-agent chemotherapy that was selected either by a medical oncologist or an in vitro human tumor cloning system.[14] Of the 246 patients who received an assay-determined drug, 62 (25%) responded to chemotherapy versus 50 of 358 patients (13%) who received a clinician-picked reagent. This author subsequently performed a similar study, this time randomizing patients with advanced metastatic cancer to receive single-agent chemotherapy selected either by a medical oncologist or an in vitro capillary tube cloning system.[15] Among the patients whose drug was selected by the clinician, 1 of 36 (3%) had a tumor response. Among the patients whose drug was selected by the capillary system, 4 of 19 (21%) had tumor responses. Although this assay resulted in improved response rates, it did not translate into prolonged survival for these patients. Other investigators have also demonstrated improved response rates, but there is little evidence demonstrating prolonged survival by using chemosensitivity assays.[16,17]

The studies by Von Hoff et al. attempted to predict the "clinical sensitivity" to any cytotoxic agent. In contrast, the assay we employed is a chemoresistance assay. This extreme drug resistance assay developed by Kern et al. has demonstrated up to 99% accuracy at identifying drugs that will be clinically ineffective.[6] Investigators have shown that response rates to chemotherapy are improved when patients receive drugs to which their tumors were not resistant in vitro,[18] and it has been demonstrated that using this assay to direct chemotherapy can improve survival.[19]

Kern and Weisenthal studied clinical responses to chemotherapy in 450 patients who had in vitro chemoresistance testing.[6] There was only 1 of 127patients (0.8%) with extreme in vitro chemoresistance who clinically responded to chemotherapy. Among patients with low in vitro resistance, clinical responses varied from 20% in the sarcoma group to 64% in the colon cancer group. However, a 52% overall response to chemotherapy was seen among all patients with low in vitro resistance. Clinical responses to chemotherapy were significantly greater among patients who had low in vitro drug resistance versus those with intermediate or extreme drug resistance.

Loizzi et al. used results produced from the same in vitro chemoresistance assay to direct chemotherapy in patients with recurrent ovarian cancer.[18] These authors compared the responses of 50 patients who received assay-guided therapy with 50 patients who received empiric chemotherapy. Among 62 women with platinum-sensitive disease, overall responses were 42% in the assay-guided group and 16% in the empiric group. Median survival was 38 months in the assay-guided group and 21 months in the empiric group.

Mehta et al. performed a double-blinded retrospective analysis of 96 patients comparing in vitro drug resistance to overall survival in patients with breast cancer.[19] Survival in patients who received CMF (cyclophosphamide, methotrexate, fluorouracil) was compared with their in vitro responses to 4HC and 5-FU. Survival in patients who received AC [doxorubicin (Adriamycin) and cyclophosphamide (Cytoxan)] was compared with their in vitro responses to 4HC and doxorubicin. There was a 5-year survival rate of 45% among patients whose treatment drugs had high in vitro chemoresistance, whereas women who were treated with drugs that had low chemoresistance experienced a 5-year survival rate of 81%.

Although most antineoplastics are ineffective in carcinoid patients, 5-FU and doxorubicin are drugs that have generated clinical responses. Therefore, it was not unexpected that 5-FU and doxorubicin had the highest incidence of low chemoresistance among all reagents tested. However, we were surprised that nearly 70% of the specimens tested with these reagents demonstrated low chemoresistance to their use. Kern et al. studied a diverse patient group with multiple types of tumors.[16] However, among this diverse group, 52% (range 20–64%) of patients with low in vitro drug resistance responded to chemotherapy. If we could generate a similar 52% clinical response rate in the group of carcinoid patients that had low in vitro chemoresistance (approximately 70% of patients treated with doxorubicin and 5-FU), then we could potentially see a response in 35% (52% of the 70% with low resistance) of all carcinoid patients treated with these reagents. This would be an improvement over the current standards. Perhaps even further improvements could be achieved if combinations of multiple low-resistance reagents were used in selected patients.

Studies have demonstrated that response rates to chemotherapy and survival are improved when patients receive drugs to which their tumors have low chemoresistance in vitro. We demonstrated that low in vitro resistance to chemotherapeutics was highly prevalent among typical carcinoids, while extreme drug resistance was comparatively infrequent. This implies that there may be less clinical chemoresistance and more chemosensitivity among typical carcinoid tumors than studies have previously revealed. We feel that our findings warrant additional investigations assessing the response of carcinoid tumors to assay-guided chemotherapy regimens.

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